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ORIGINAL PAPER

Preparation and characterization of a novel injectable in situ cross-linked hydrogel

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Abstract A novel injectable in situ cross-linked hydrogel was prepared from α,β-poly(*N*-hydroxyethyl)-DL-aspartamide (PHEA), which had excellent biocompatibility and biodegradability. PHEA was modified by acryloyl chloride (AC) via the reaction between hydroxyl groups and acryl groups to introduce reactable double bonds into the chain. Two macromers with different degrees of derivatization were prepared. Through NMR and FT-IR characterizations, the structures of the polymers were proposed. Hydrogels were prepared by covalent cross-linking between double bonds with a transition from liquid to gel at body temperature. The effect factors on gelation time, swelling ratio and gel content were investigated in detail. It was found that the grafting ratios of AC, concentrations of macromer and initiator had great influence on the gelation time. And it could be adjusted to meet the requirements of an injectable material.

Keywords Injectable hydrogel · In situ cross-linked · Polysuccinimide · Gelation time

Introduction

Hydrogels are cross-linked, three-dimensional polymer network. They can be used in fields of agriculture [1], pharmacy [2–4], food industry [5], chemistry [6] and especially, clinical and medical fields [7–9] because of their excellent

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biocompatibility, hydrophilicity and closely resemble natural living tissues. Injectable in situ cross-linked hydrogels are increasingly attractive in clinical applications, for example, controlled release of drugs [10, 11], scaffolds used in tissue engineering [12, 13], wound healing [14, 15] and so on. Hydrogels composed of collagen [16, 17], chitosan [18] gelatin [19, 20] and poly(propylene fumarate) [21, 22] are widely applied as the injectable scaffolds. They can be easily injected to the wanted sites, solidified in situ and conform to the shapes of the wanted sites with minimal invasive procedure of injection. In situ cross-linked hydrogels prepared from polymers cross-linked by noncovalent bonds such as ion bonds, electrostatic and hydrophobic interactions have been investigated a lot [23]. But for clinical applications, it is important that the in situ cross-linked hydrogels should be stable, while noncovalent bonds mentioned above are not, especially under the environment of a body medium. To develop novel drug carriers or tissues scaffolds, chemically cross-linked hydrogel are investigated widely. This kind of hydrogel is much stable and could allow the potential for a broad degree of control over the degradation kinetics of the gel. Researches have been done about photocross-linked hydrogels [24, 25], but photopolymerization cannot be carried out uniformly in a large or thick system, especially in many clinical applications in which the penetration depth is quite limited and light distribution is inhomogeneous. To address the problem, redox initiator systems have been employed to initiate the cross-linking reaction, such as ammonium persulfate (APS)/*N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) system which is water soluble and has neutral pH during the course of gelation.

In this work, we reported the preparation and characterization of a novel injectable in situ chemically cross-linked hydrogel based on α,β-poly(*N*-hydroxyethyl)-DL-aspartamide (PHEA), a modified product of polysuccinimide (PSI) which came from a natural amino acid. PHEA is biocompatible, biodegradable and water soluble. It is often used as a stating material for various biomedical and pharmaceutical applications [26–28]. In the text, PHEA was modified subsequently with acryloyl chloride (AC) to introduce reactable double bonds into the chains and the macromer was indicated as PHA. The transition from the state of liquid to gel could be accomplished via radical polymerization after being injected into the body. To evaluate the feasibility as injectable in situ cross-linked hydrogels, the gelation time, swelling ratio, gel content and other physicochemical characterizations were characterized. By varying the conditions of the gelation system, the properties could be varied allowing the system to be used in a number of applications.

Materials and methods

Materials

Polysuccinimide, ethanolamine, *N*,*N*-dimethylform amide (DMF), acetone, triethylamine, AC, 1-butanol, *N*,*N*′-methylenebis acrylamide (BIS), APS, TEMED.

Synthesis and characterization of PHEA

α,β-Poly(*N*-hydroxyethyl)-DL-aspartamide was prepared from PSI, obtained by thermal polycondensation of D,L-aspartic acid. The procedure was reported by Giammona [29]. Briefly, 10 g PSI was dissolved in 50 mL of DMF in a 100-mL baker, and then ethanolamine (6.3 mL) was added to the solution. The reaction was kept for 48 h at 40 °C. The reaction solution was precipitated in excessive acetone, filtered and dried under reduced pressure at 60 °C. The product was dissolved in deionized water, ultrafiltered to remove the impurity and dried again.

Synthesis and characterization of AC derivatization of PHEA

Acryloyl chloride derivatization of PHEA (PHA) was prepared by the reaction of PHEA and AC in the present of triethylamine. Four grams of PHEA was dissolved in 80 mL DMF in a 250-mL baker with a magnetic stirring. The solution was transferred into a 250-mL round bottom flask equipped with a mechanical stirring device and a tap funnel. The dissolved oxygen was removed by passing nitrogen gas for 15 min. Triethylamine used as catalyst was added to the mixture. After the flask was cooled to 0 °C in an ice bath, the mixture solution composed of suitable amount of AC and 10 mL of DMF was added dropwise. The reaction mixture was stirred for 24 h at room temperature. Finally, the insoluble triethylamine salt was filtered. The filtrate was precipitated in excessive 1-butanol, filtered, washed several times with acetone and freeze-dried. The product was dissolved in deionized water, ultrafiltered to remove the impurity, and freeze-dried. This macromonomer was called PHA denoted acrylate-grafted macromonomer with PHEA as main chain.

Characterizations of polymers

Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy spectrums were recorded as pellets in KBr in the range 4000–400 cm^{-1} using a Fourier Transform Infrared Spectrum analyzer 3100FTIR, Varian, USA.

Nuclear magnetic resonance analysis

^1H nuclear magnetic resonance (^1H NMR) spectroscopy was used to identify the synthesis of PHEA and PHA. The samples were analyzed with an AC-80MHZ spectrometer; spectrums were recorded in D_2O, and tetramethylsilane was used as internal standard.

Gel permeation chromatography

The molecular weight of polymer was measured in a buffer containing 0.02 M H_3PO_4 using gel filtration chromatography by a GF-510 HQ column (Asahi Chemical Industry Co., Ltd) and a detector was at UV 206 nm (SHIMADZU,

10-ATVP), the molecular weight marker was dextran which was gift from Uppsala University (Sweden).

Specific viscosity determined by Ubbelohde viscometer

Specific viscosity, η_{sp}, of PHA was determined in deionized water measuring relative viscosities in the range 0.5–10 mg/mL at 25 °C.

Preparation of hydrogels

PHA aqueous solution was gelated by radical polymerization under the initiation of a redox system including oxidant APS and reducer TEMED. APS and TEMED were previously made into 1 mol/L solutions, respectively. The macromer PHA was dissolved in a BIS solution. Then TEMED and APS were added, whose mole ratio was kept same for all the experiments. The test tube was shaken to mix the solution after each ingredient was added. The mixture changed its state from liquid into hydrogel via being incubated at 37 ± 0.5 °C in a thermostatic water bath.

Gelation time of the hydrogel

To determine the gelation time, the test tube inverting method was employed [30, 31]. Briefly, the mixture solution composed of macromer, cross-linker, initiator and reducer was poured into a glass tube, which was tightly screw-capped. The test tube was immersed in a thermostatic water bath controlled at 37 ± 0.5 °C. After some reaction time, the solution was monitored by the inversion of the glass tube. The gel phase was defined as non-flowing gel when the hydrogel solution in the test tube was inverted.

Swelling ratio of the hydrogels

For measuring the swelling ratio, hydrogels were prepared by gelling of the mixture solutions with different components for 24 h at 37 °C. The hydrogels were balanced in PBS for 24 h at 37 °C and weighted (W_1), then dehydrated under reduced pressure at 50 °C to constant weights (W_2). The swelling ratio of the hydrogel was defined as followed:

$$\text{Swelling ratio} = \frac{(W_1 - W_2)}{W_2}\,\text{g/g} \tag{1}$$

Gel content of the hydrogels

The mixture solutions with different components were gelated at 37 °C for 24 h. The hydrogels were freeze-dried and weighted (W_3). Then the freeze-dried hydrogels were immersed in deionized water for 24 h. Finally, the hydrogels were freeze-dried again to constant weights (W_4). The gel content was defined as followed:

$$\text{Gel content} = \frac{W_4}{W_3} \times 100\% \quad (2)$$

Results and discussion

Synthesis of the macromers

α,β-Poly(*N*-hydroxyethyl)-DL-aspartamide, prepared from PSI, was chosen because of its excellent biocompatibility, biodegradability and water-solubility. It contained abundant hydroxyl groups, through which polymerizable groups could be conveniently introduced. The weight average molecular weight of PHEA, determined by gel permeation chromatography, was 66 kDa ($M_w/M_n = 1.79$). Spectroscopic data (NMR) was in agreement with the values reported in the literature [32]. In the present work, AC was grafted on the PHEA chains via the acrylation of hydroxyl groups and acryl groups (Fig. 1). The by-products and unreacted monomers were removed by ultrafiltering.

Figure 2 showed the FTIR spectrums of PSI (a), PHEA (b), PHA (c) and PHA hydrogel (d). As was seen, the absorption band at 1,813, 1,714 and 1,391 cm^{-1}

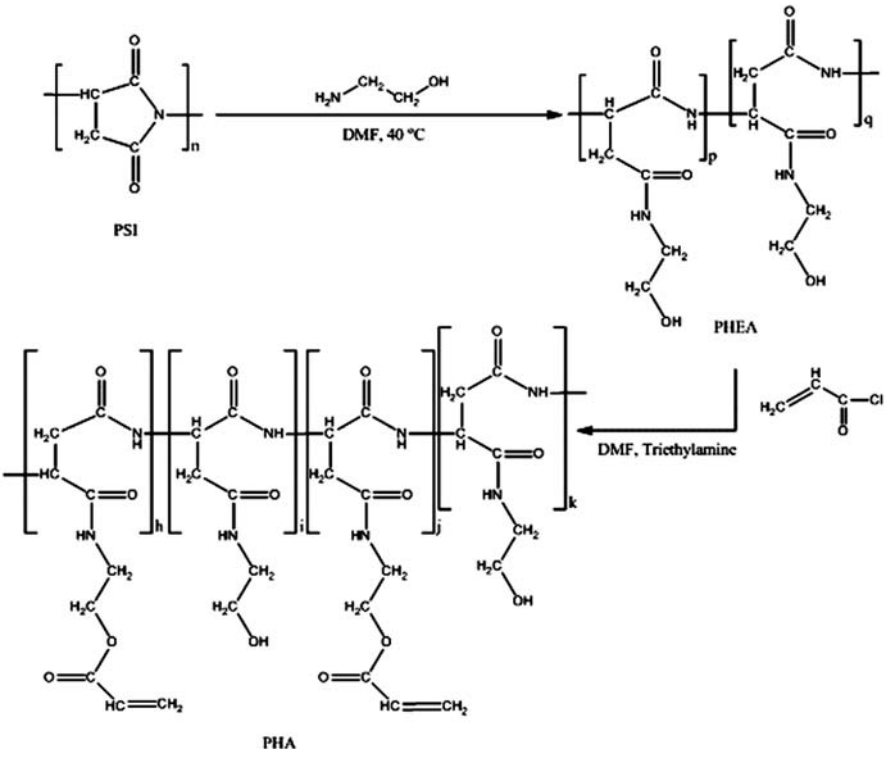

Fig. 1 The synthesis route of PHEA and PHA

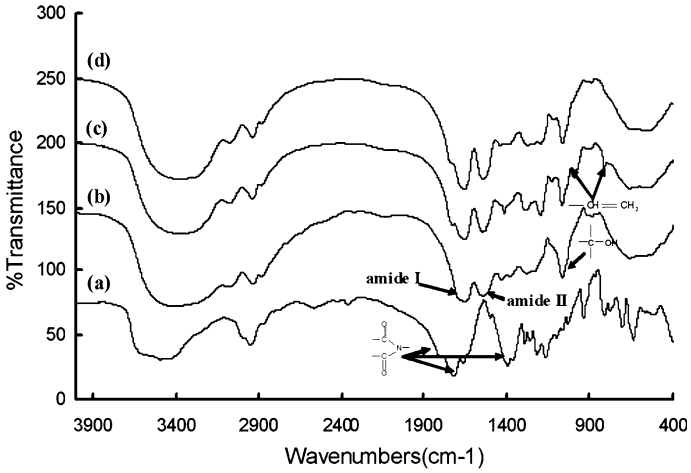

Fig. 2 Fourier transform infrared spectroscopy spectrums of **a** PSI, **b** PEHA, **c** PHA and **d** PHA hydrogel

assigned to imide became much weaker while strong peaks of –NH– (1,648, 1,543 cm^{-1}) and –OH (1,062 cm^{-1}) presented in the spectrum of PHEA. Compared the spectrum of PHEA, characteristic peaks (987 and 811 cm^{-1}) revealed that reactable groups (–C=C–) were introduced into PHA chains successfully while the absorption band of hydroxyl was observed to be smaller.

The degree of derivatization (DD) of acryloyl group was significantly influenced by the feed mol ratio of acryloyl groups in AC and hydroxyl groups in PHEA. As the molar ratio of AC increased, the DD increased. DD was determined by ^{1}H NMR (Fig. 3) and was calculated by the following ratio:

$$\mathrm{DD} = (\text{acryloyl groups}/\text{PHEA repeating unit}) \times 100(\text{mol})$$

Degree of derivatization was calculated by comparing the integral of the peak related to protons at 6.2 ppm assigned to –*CH*=CH_2 with the integral of the peak related to protons at 2.8 ppm assigned to –NH–CH(CO)–*CH_2*–. Feed mol ratio, DD, Molecular weight and M_w/M_n of macromers were shown in Table 1.

Preparation of the hydrogel

As acryloyl groups were introduced into the macromer chains, the hydrogel described here was formed via cross-linking reaction between double bonds. To make sure the accomplishment of the cross-linking reaction at body temperature, a redox system (APS/TEMED) was used to initiate the reaction. An appropriate amount of BIS was used as cross-linker, and its concentration was kept very low in the experiments, which was at 4 mg/mL. Both the initiate system and cross-linker were widely employed in the preparation of hydrogel for clinical and pharmacological investigation [33–35]. The mixture was incubated at 37 °C after the addition of APS/TEMED. The liquid solution transformed into hydrogel which could sustain its macroscopic shape in a few minutes. In the FTIR spectrum of PHA hydrogel

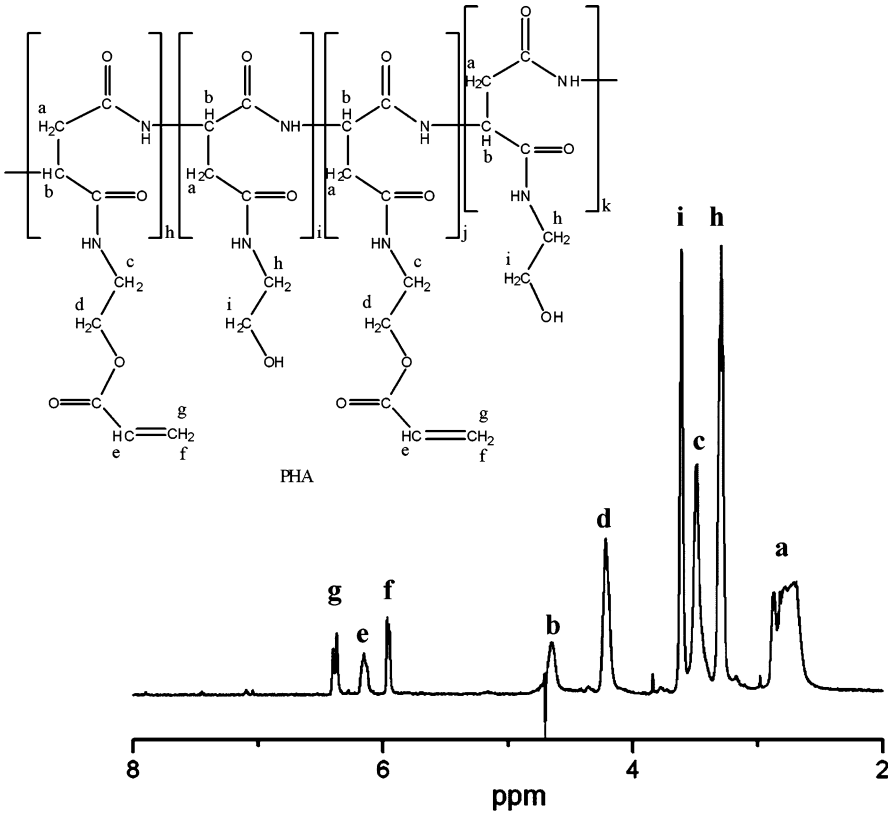

Fig. 3 ^{1}H Nuclear magnetic resonance spectrum of PHEA

Table 1 Synthesis and composition of macromers

Sample	Feed mol ratio (PHEA repeat unit:AC)	DD%	Weight average molecular weight, kDa (M_w)	Polydispersity index (M_w/M_n)
PHA1	1:1.5	19.6	64	1.82
PHA2	1:3	31.4	53	1.84

(Fig. 2d), the absorbance at 987 and 811 cm^{-1} (–C=C– double bonds) had disappeared, indicating the thorough occurrence of the cross-linking between macromer and cross-linker and preliminarily demonstrating as prepared PHA had the potential capability to be used as a novel injectable in situ cross-linked material. Figure 4 illustrated the macroscopic gelation process.

Adjustment of the gelation time

As an injectable material, an appropriate gelation time was very important. It should not be very long to prevent from the diffusion of the injected solution to the

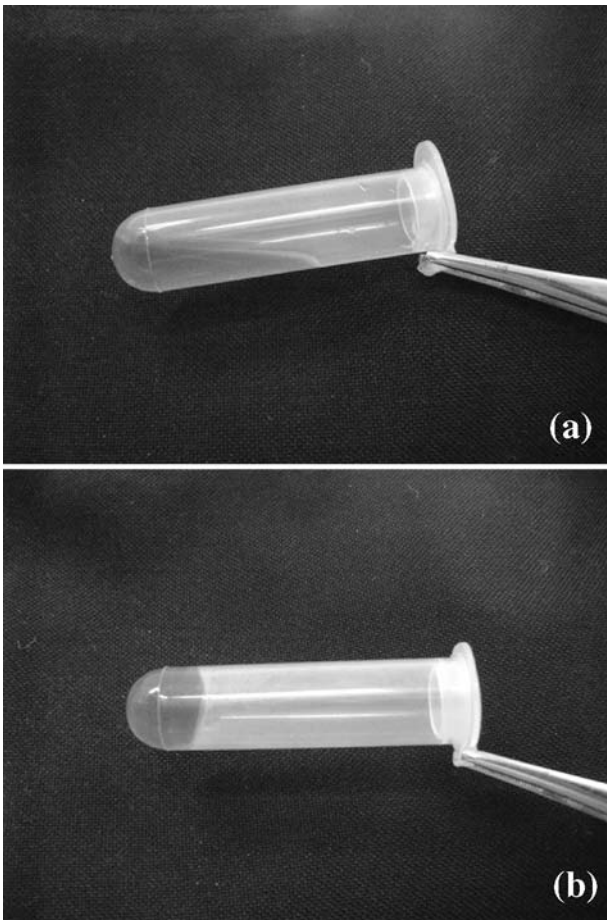

Fig. 4 The macroscopic gelation process: **a** solution of macromer, cross-linker and initiator; **b** the PHA hydrogel

surrounding tissue ahead of hydrogel formation and favor the shape persistence. On the other hand, it should not be very short to benefit the operation. Fortunately, the gelation time was highly adjustable in our experiments.

Although the generation and diffusion of free radicals and the motion ability of the macromer chains would be enhanced at higher temperature because of the temperature sensitivity of APS/TEMED system, which led to quicker gelation, the hydrogel was expected to form at body temperature. So compared with rising the temperature, varying the concentration of the initiator was chosen as one method to control the gelation time.

Figure 5 showed the effect of initiator concentration on the gelation time. A higher initiator concentration resulted in a shorter gelation time. When the concentration was set at 8 and 24 mmol/L, formation of hydrogel required only 520 and 70 s, respectively.

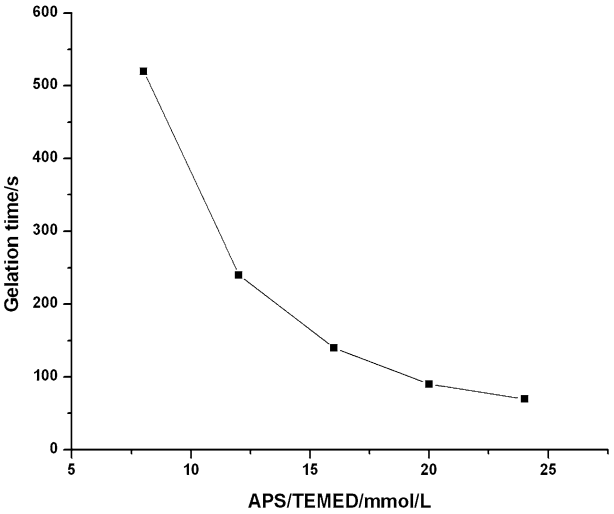

Fig. 5 Gelation time of 0.1 g/mL PHA solution as a function of APS/TEMED concentration at 37 °C

At the same concentration of initiator and cross-linker, higher concentration of maromer led to shorter gelation time (Fig. 6), and this was presumable attributable to the higher molar ratio between double bonds and initiator. While the gelation time could not be shorten at relative high concentration of macromer, which due to the increasing viscosity as a function of macromer's concentration. The diffusion of free radicals and the motion ability of the macromer chains would be depressed in this situation. This phenomenon could also be seen with DD increasing (Fig. 6).

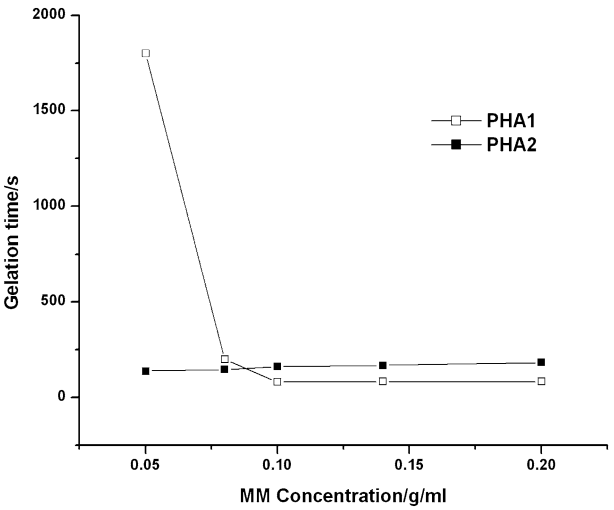

Fig. 6 Gelation time of PHA solutions as a function of macromer concentration at 37 °C (ASP/TEMED: 16 mmol/L)

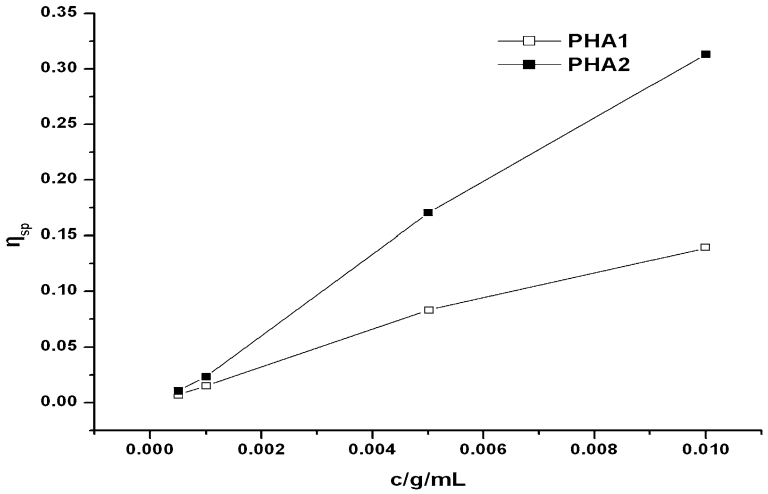

Fig. 7 η_{sp} of PHA1 and PHA2 solution as a function of macromer concentration

The macromer with higher DD (PHA2) gelated much quicker than that with lower DD (PHA1) at relatively low marcomer concentration because of the high molar ratio between double bonds and initiator. But it was not shortened with increase of PHA2 concentration. In Fig. 7, the η_{sp} of PHA2 solution rose quickly with increase of PHA2 concentration, which restricted the motion ability of the macromer largely. As a result, it took a longer time to gel at higher concentration.

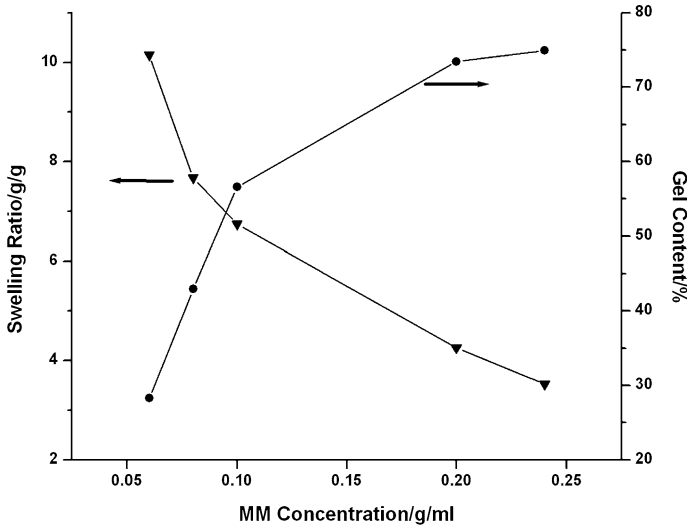

Fig. 8 Swelling ratio and gel content of PHA hydrogels as a function of PHA concentration

The swelling ratio and gel content of the hydrogel

Figure 8 presented the swelling behavior in PBS and the gel content of the hydrogels formed at 37 °C with different PHA concentration. The gel content is a basic parameter to calibrate gel formation due to cross-linking because not all the macromonomers eventually join the gel network.

The swelling ratio matched inversely with the gel content of the hydrogels. Hydrogels with higher gel content had lower swelling ratio which was largely restricted by the cross-linking density. Cross-linking of the macromonomers was largely dependent on the encountering probability of –C=C– double bonds. Cross-linking reaction could take place only if a macromolecular radical was close enough to another –C=C– bond. Higher PHA concentration led to more –C=C– bonds. As a result, there will be a higher chance for the macromolecular radicals to react with other –C=C– bonds. Hence, the gel content showed a positive correlation with the PHA concentration.

Conclusions

A novel injectable in situ cross-linked hydrogel was developed and characterized based on PHEA, which was biocompatible, biodegradable and water soluble. PHEA was modified by AC to introduce reactable double bonds into the chain. It could be injected with the state of liquid and underwent a transition to gel at body temperature. This transition was accomplished by covalent cross-linking between double bonds, which was more stable and controllable. The gelation time could be controlled in the range of 1 min to tens of minutes via adjusting the components. This gelation behavior makes the hydrogel more promising and attractive materials for biomedical applications such as drug delivery, cell encapsulation or tissue engineering.

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